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# Overexpression of the Proto-Oncogene C-jun in Association With Low-Risk Type Specific Human Papillomavirus Infection in Condyloma Acuminata

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Infection with different types of human papillomavirus (HPV) is associated with neoplasia at different anatomic sites. The "low-risk" HPVs (LR-HPV) are responsible for benign genital lesions such as condyloma acuminata. In order to clarify the tumorigenic mechanism of LR-HPV, the HPV infection status was investigated and the expression of the c-jun proto-oncogene in different HPV-related skin and genital lesions analyzed. Of the 17 condyloma specimens analyzed by Western blotting, 13 cases (76.5%) exhibited overexpression of the c-jun gene. All 13 cases harbored high copy numbers of the LR-HPV genome with an average of 926 copies per cell, whereas the other four cases had an average of 12 copies of LR-HPV per cell ($P < 0.001$). Further typing of HPV by Southern blotting revealed that HPV-6 and HPV-11 infections predominated in c-jun positive cases. The c-jun protein was detected much less frequently in cervical cancers (three of 29, or 10.3%) and skin warts (one of 10), and was not detected in five genital polyps or in five normal cervical tissues. These findings suggest a type 6/11-specific induction of c-jun gene expression in HPV-related neoplastic lesions. 

**KEY WORDS:** HPV typing, genital warts, cervical cancer, skin warts

## INTRODUCTION

There is compelling evidence showing that genital human papillomaviruses play major roles in the development of benign and malignant genital neoplasia. Of the 70 different types of HPVs that have been discovered, about 20 are associated with ano-genital lesions. The other types of HPVs have been associated mostly with cutaneous lesions such as skin warts [de Villiers, 1989, 1994]. Infection with the "low-risk" genital HPVs (type 6, 11, 42, 43, 44, etc.) is responsible for condyloma acuminata and laryngeal papilloma [Gissmann et al., 1983; Lorincz et al., 1992]. The "high-risk" viruses such as HPV-16 and HPV-18 are associated with cervical intraepithelial neoplasia (CIN) and invasive cervical cancer [Dürst et al., 1983; Boshart et al., 1984]. The transformation activities of HPV-16 and HPV-18 require the expression of the E6 and E7 genes which, either individually or in combination, can immortalize human foreskin and cervical keratinocytes [Munger et al., 1989; Hawley-Nelson et al., 1989; Watanabe et al., 1989]. In the genital condyloma, however, the major transcripts of HPV-6 and HPV-11 encodes E1 ^ E4, E5a and E5b proteins with little expression of E6 and E7 [Chow et al., 1987]. The E5a gene of HPV-6 and HPV-11 has been found to be capable of transforming NIH 3T3 cells and human keratinocytes [Chen and Mounts, 1990; Tsao et al., 1994b]. Recently, we found that the E5a gene of HPV-11 is capable of initiating transformation of NIH 3T3 cells, but is dispensable for the maintenance of the transformed phenotypes [Chen et al., 1994a]. Interestingly, the expression of the proto-oncogene c-jun was found to be constantly activated during E5a induced cell proliferation, and the cell transformation could be reverted by introduction of an antisense oligonucleotide of c-jun [Chen et al., 1994b]. Therefore, the E5a protein of HPV-11 appears to be an important "tumor initiator" which renders the cells susceptible to uncontrolled proliferation in which the proto-oncogene c-jun may play an important role.

The expression of some proto-oncogenes are induced quickly in response to growth factors and mitogens, as the primary early effectors in the signal transduction pathway. Products of the jun and fos gene families represent the major early effectors which, in the form of homo- or hetero-dimers, constitute the AP-1 transcription factor [for a review, see Cooper, 1990]. When constitutively

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activated, usually by the transduction of retroviruses, members of the jun gene family such as c-jun, jun-B and jun-D confer a transformation phenotype to primary cultured animal cells [Schutte et al., 1989; Maki et al., 1987]. To understand the significance of induction of c-jun gene expression in HPV-11 E5a transformed cells, we studied the expression status of c-jun in HPV-related neoplastic lesions.

In this study, tissues from condyloma acuminata, invasive cervical cancer, and skin warts were examined for HPV infection and c-jun expression. In addition, the HPV type was determined in HPV positive cases. Specific overexpression of the c-jun gene was observed in cases of condyloma acuminata that were infected with HPV-6 and HPV-11.

## MATERIALS AND METHODS

### Clinical Specimens, Extraction of Genomic DNA and Proteins

Specimens were obtained from the Obstetrics/Gynecology, Dermatology and Urology clinics of the Tri-Service General Hospital, Taipei, Taiwan. They were prepared by snap-freezing in liquid nitrogen and stored at −70°C until use. Part of each sample was examined pathologically for diagnosis. Genomic DNA was prepared by proteinase K digestion and phenol extraction. Tissue fragments were digested overnight at 55°C with 1 mg/ml of proteinase K. Lysates were extracted with phenol/chloroform (1:1 and with 4% isoamyl alcohol), and the DNA in the aqueous phase was precipitated with isopropanol, washed with 70% ethanol and resuspended in TE buffer. The concentration of the genomic DNA was determined from the absorbance of UV light at 260 nm. Protein was extracted by homogenizing tissue in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.5% SDS, 50 mM Tris-HCl, and 1 mM phenylmethylsulfonyl fluoride). Protein was recovered from the supernatant after centrifugation. The concentration of protein was determined by the bicinchoninic acid (BCA) detection assay (Pierce) [Smith et al., 1985].

### Quantitative Detection of HPV DNA With the Hybrid Capture System

The Hybrid Capture system (Digene) was used to detect the presence of HPV DNA of either the high-risk (type 16, 18, 31, 33, 35, 45, 51, 52, 56) or low-risk (type 6, 11, 42, 43, 44) types with modifications for quantitative assay. Briefly, 5 μg of genomic DNA from each specimen was denatured and hybridized with a pool of RNA probes derived from either high-risk or low-risk HPV types. The DNA-RNA hybrids were then "captured" by an alkaline phosphatase conjugated antibody coated in a glass tube. The enzymatic activity was then detected by a chemiluminescence method through a luminometer. A panel of copy number controls consisting of 0.1, 1.0, 10, 100, and 1,000 HPV copies per cell were analyzed together with the samples. One copy of HPV per cell genome is equal to 6.67 pg of HPV DNA in 5 μg of genomic DNA, as calculated from the genomic size of HPV (8,000 kb) and a human cell ($6 \times 10^9$ bp). In practice, 10 pg of pBR322 cloned HPV-11 and HPV-16 DNA were reconstituted in 5 μg of herring sperm DNA to produce the one copy per cell control. A regression coefficiency of more than 0.996 was typically achieved for both LR- and HR-HPV assays with a sensitivity of up to 0.1 copy per cell. The cross reactivities between the HR- and LR-HPV probes and the DNA samples were also determined. Whereas the HPV-11 DNA reacted with (as more than one copy per cell) a HR-HPV probe at a concentration of about 1,000 copies per cell, the HPV-16 DNA cross reacted with the LR-HPV probe at about 100 copies per cell. The coefficients of variation of the triplicate tests of both the positive (a one copy per cell standard) and negative (5 μg of herring sperm DNA) controls were typically less than 20% and the positive to negative mean ratios were greater than 1.5.

### HPV Typing by Southern Blot Hybridization

Methods of Southern blot hybridization for HPV detection and typing have been published [Chen et al., 1993b; Tsao et al., 1994a]. Briefly, 5 μg of genomic DNA was digested with EcoRI or Hpa I restriction enzymes and resolved by agarose gel electrophoresis. The DNA was transferred onto nitrocellulose membrane, denatured with 0.3 M NaOH and neutralized with 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.4). A full length HPV-11 DNA probe was used for hybridization in a solution of 50% formamide, 5× SSC, 1× Denhardt's solution, 0.2% SDS and 150 μg/ml of sheared salmon sperm DNA at 42°C. After hybridization, filters were washed twice with 1× SSC at 55°C for 1 hour. The HPV probe was prepared by restriction digestion and gel purification (Gene Clean, *BIO* 101) of a pBR322 plasmid containing the HPV-11 genome, and labelled with the Photogene chemiluminescence system (BRL). The hybridized products were detected by exposing membranes to X-ray film (Kodak). One copy of HPV-11 DNA per cell could be detected (Fig. 2).

### Western Blot Analysis

The extracted tissue proteins were mixed with 1/10 volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. After boiling for 10 min, 10 μg of each protein lysate was separated by SDS-PAGE. The resolved proteins were transferred onto nitrocellulose membranes, reacted with a rabbit polyclonal anti-c-jun antibody (Oncogene Science) and alkaline phosphatase (AP) conjugated goat anti-rabbit Ig G, and visualized by the Western Blue stabilized for AP (Promega). HPV-11 E5a gene transformed NIH 3T3 (E5-3T3) cells which highly express c-jun [Chen et al., 1994a] were used as a positive control. The densitometric intensity of the 39 kd c-jun band from the Western analysis of the E5-3T3 cell extracts was used as a scale standard. An intensity equivalent to 1.0–5.0 μg of E5-3T3 cell extract was defined as "++," that equivalent to 0.1–1.0 μg of extract as "+," and that equivalent to less than 0.1 μg extract as "+/−."

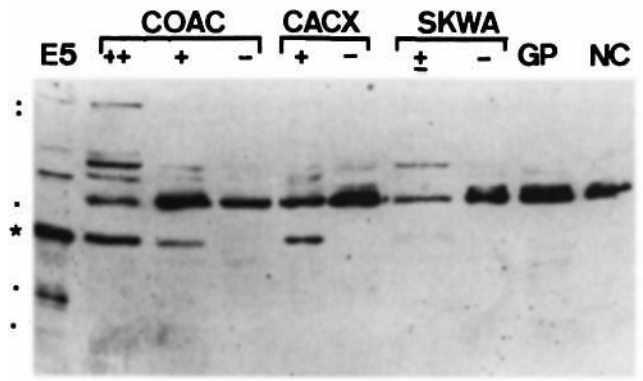


Fig. 1. Western blot analysis of c-jun oncoprotein in HPV-related clinical specimens. The expression of c-jun in representative cases of condyloma acuminata (COAC; M235, M377, M162, from left to right), cervical cancer (CACX; M558, M85), skin warts (SKWA), genital polyps (GP) and normal cervix (NC) was analyzed by Western blotting with an intensity scale of the 39 kd c-jun band (denoted by an asterisk) from "−" to "++" as defined in the text. Ten micrograms of protein extracts from each sample and the HPV-11 E5a gene transfected NIH3T3 cells (E5) was analyzed. The protein size markers of 106, 80, 49, 32, and 27 kds are indicated as dots.

## RESULTS

### The Proto-Oncogene C-jun Is Frequently Overexpressed in Condyloma Acuminata But Not in Other HPV-Related Benign or Malignant Tumors

The specimens collected for examination included 17 condyloma acuminatas of the genitalia of both sexes, 29 squamous cell cervical carcinomas, 10 skin warts (four verruca vulgaris, three verruca plantaris, and three verruca plana), five genital (three endocervical and two endometrial) polyps and five normal cervix samples. The expression of c-jun proto-oncogene in each specimen was analyzed by Western blot analysis. The 39 kd c-jun protein was detected in 13 of 17 (76.5%) condyloma acuminata samples (Fig. 1, Table I). However, only three of 29 (10.3%) cervical cancers (Table II), one of 10 skin warts, and none of the five genital polyps or five normal cervical tissues expressed the c-jun protein (Fig. 1, Table III). The ratio of c-jun expression in condyloma acuminata samples was significantly higher than in the other samples ($P < 0.001$ by chi square test).

### The Overexpression of C-jun Is Associated With High Levels of LR-HPV Infection

In order to evaluate the status of HPV infection, the specimens were tested first for the presence of HPV DNA of either high-risk (HPV-16, 18, 31, 33, 35, 45, 51, 52, 56) or low-risk (HPV-6, 11, 42, 43, 44) HPV DNA with the Hybrid Capture system. The results are shown in Tables I and II and are summarized in Table III. The low-risk group of HPV DNA was detected at a level of more than one copy per cell in 16/17 (94.1%), 2/19 (10.8%), 1/10 (10%), 0/5 and 0/5 specimens of condyloma acuminata, cervical cancer, skin warts, genital polyps and normal cervix, respectively, whereas the high-risk group of HPV DNA was detected in 2/17 (11.8%), 13/19 (68.4%), 2/10 (20%), 0/5 and 0/5 specimens. The dosage of LR-HPV infection in the condyloma cases varied from six to 2,541 copies per cell (Table I). All of the 13 condyloma samples that expressed c-jun harbored more than 147 copies of LR-HPV per cell (mean 926), whereas the other four condyloma samples which did not express c-jun harbored less than 22 copies of LR-HPV per cell (mean 11.9) (Table I, $P < 0.001$). In the cervical cancer samples, only one of the three cases that expressed c-jun also harbored LR-HPV. The overexpression of c-jun in condyloma acuminata samples appears to be closely related to high levels of LR-HPV infection, whereas it appears to be an independent event in cervical cancer.

### Both HPV-6 and HPV-11 Infections Are Associated With the Overexpression of Proto-Oncogene C-jun

We have shown previously that the HPV-11 E5a gene product can induce c-jun gene expression in vitro. To determine whether induction of c-jun is also associated with other LR-HPV types, we determined the HPV types of eight LR-HPV infected condyloma cases by restriction fragment length polymorphism and Southern blot analysis. Of the eight cases, five harbored HPV-6 DNA and three harbored HPV-11 DNA (Table I, Fig. 2). As a comparison, the three c-jun expressing cervical cancer cases harbored only significant levels of HPV-16 or HPV-58 DNA (Table II).

## DISCUSSION

Results of the present study reconcile the in-vitro results and indicate an association of c-jun overexpression with infections of both HPV-6 and HPV-11, but not with infection of high-risk types of genital HPVs or cutaneous HPVs. Overexpression of c-jun was noted in a much higher proportion of LR-HPV related condyloma samples (76.5%) compared with HR-HPV related cervical cancer (10.3%), non-genital HPV related skin warts (10%), or non-HPV related genital polyps (0%). LR-HPV, differing from other types of HPV, appears to be involved in a tumorigenic mechanism involving the activation of the c-jun gene. The expression of c-jun was exclusively observed in condyloma samples harboring high copy numbers of LR-HPV, suggesting an association of c-jun induction and high levels of LR-HPV infection. The induction of c-jun in LR-HPV infected condylomas seems unlikely to be a general effect of cell proliferation since c-jun was not detected in any of the five negative genital polyps.

Of the three cervical cancers which expressed c-jun, HPV-58 and HPV-16 DNA were detected in various amounts, but no LR-HPV DNA was detected. The occasional overexpression of c-jun in cervical cancers seems to be independent of HPV infection. Interestingly, whereas most low-risk HPVs are present in the condyloma cells as an episomal form, a special case of a cervical cancer which only harbored an integrated form of HPV-11 [Chen et al., 1993a] did not express c-jun. Whether

TABLE I. Status of HPV Infection and c-jun Expression in Condyloma Acuminata Tissues

| Patient | No. of HPV copies per cell | | C-jun[b] | HPV typing[c] |
|---|---|---|---|---|
| | HR-HPV[a] | LR-HPV[a] | | |
| M271 | 2.9 (CR) | 2541 | ++ | HPV-6 |
| M70 | 3.4 (CR) | 2399 | ++ | HPV-6 |
| M124 | 0.4 | 892 | ++ | HPV-6 |
| M3 | 0.3 | 749 | ++ | HPV-11 |
| M116 | 0.9 | 953 | ++ | HPV-11 |
| M550 | Neg | 628 | ++ | Not done |
| M235 | Neg | 355 | ++ | Not done |
| M167 | Neg | 148 | ++ | HPV-11 |
| M234 | 9.2 | 467 | ++ | Not done |
| M273 | 0.3 | 511 | + | Not done |
| M377 | 1.8 (CR) | 1767 | + | HPV-6 |
| M26 | Neg | 279 | + | HPV-6 |
| M228 | Neg | 339 | + | Not done |
| M160 | Neg | 20 | − | Not done |
| M241 | Neg | 22 | − | Not done |
| M97 | 14.6 | 6 | − | Negative |
| M162 | Neg | Neg | − | Not done |

[a]The HPV copy numbers per cell was determined from the luminescence signals as defined in the text. The ratio of positive control mean to negative control mean for HR-HPV and LR-HPV were 1.71 and 1.79, respectively. The coefficients of variation of positive and negative control results of the LR-HPV test were 6.6% and 1.0%, respectively, and those for HR-HPV tests were 12% and 9.3%, respectively. Cross reactivity (CR) was as defined in the text.
[b]The amount of c-jun protein was scored according to the intensity of Western banding read with a densitometer. The grades of intensity was as defined in the text.
[c]As determined by Southern blot analysis.

TABLE II. Outline of HPV Typing and C-jun Expression in Cervical Cancers

| Specimen | HR-HPV[a] | LR-HPV[a] | C-jun[a] | HPV typing[b] |
|---|---|---|---|---|
| T21 | Not done | Not done | − | HPV-16 |
| T14 | Not done | Not done | − | HPV-16 |
| T35 | Not done | Not done | − | HPV-16 |
| T31 | Not done | Not done | − | HPV-16 |
| T18 | Not done | Not done | − | HPV-16 |
| T36 | Not done | Not done | − | HPV-16 |
| T11 | Not done | Not done | − | HPV-18 |
| T20 | Not done | Not done | − | HPV-18 |
| T40 | Not done | Not done | − | HPV-33 |
| T6 | Not done | Not done | − | HPV-11 (int) |
| T15 | 0.8 | Neg | + | HPV-58[c] |
| T39 | 68.6 | Neg | + | HPV-16 |
| M558 | 10.1 | 0.7 | ++ | HPV-16 |
| M272 | 3.0 | 1.0 | − | Not done |
| M161 | 0.3 | 1.5 | − | Not done |
| M431 | 44.2 | Neg | − | Not done |
| M85 | 890.3 | 1.7 (CR) | − | Not done |
| M554 | 364.4 | 2.4 (CR) | − | Not done |
| M13 | 63.0 | 0.4 | − | Not done |
| M557 | 75.0 | Neg | − | Not done |
| M556 | 28.1 | Neg | − | Not done |
| M55 | 7.0 | 0.7 | − | Not done |
| M507 | 15.1 | 0.5 | − | Not done |
| M385 | 1.8 | 2.1 | − | Not done |
| M386 | 3.4 | Neg | − | Not done |
| M92 | 0.0 | Neg | − | Not done |
| M379 | 0.6 | 0.1 | − | Not done |
| M191 | 0.6 | Neg | − | Not done |
| M505 | 0.8 | Neg | − | Not done |

[a]See footnote to Table I.
[b]As referred to the PCR-based typing reported previously [Chen et al., 1993a]. int, integration form of HPV-11.
[c]Note that HPV-58 was not included in the cocktail of Hybrid Capture HR-HPV probe.

TABLE III. Summary C-jun Expression in Genital and Cutaneous Lesions

| | C-jun | LR-HPV[a] | HR-HPV[a] |
|---|---|---|---|
| Condyloma accuminata | 13/17 (76.5%) | 16/17 (94.1%) | 2/17 (11.8%) |
| Cervical cancer | 3/29 (10.3%) | 2/19 (10.5%) | 13/19 (68.4%) |
| Skin wart | 1/10 (10%) | 1/10 (10%) | 2/10 (20%) |
| Genital polyp | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) |
| Normal cervix | 0/5 (0%) | 0/5 (0%) | 0/5 (0%) |

[a]An HPV DNA level of more than 1.00 copy per cell in the absence of cross reactivity was considered as positive for HPV infection.

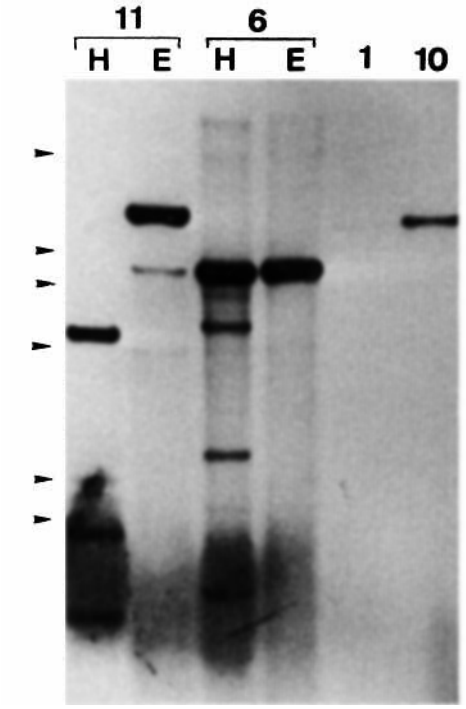


Fig. 2. HPV typing by Southern blot analysis. The presence of HPV-11 (11) or HPV-6 (6) DNA in condyloma tissues were differentiated by restriction polymorphism and Southern analysis. Five micrograms of genomic DNA from M167 (HPV-11), M70 (HPV-6) and copy number standards (1 and 10 copies per cell) was analyzed. Whereas the HPV-6 genome had single restriction sites for both HpaI and EcoRI, HPV-11 had three HpaI sites but no EcoRI site. HindIII digested lambda phage DNA markers are indicated by arrows.

the E5a gene function was disrupted in this special case deserves further investigation.

The overexpression of the c-jun early response gene in condyloma acuminata suggests a role of the AP-1 transcription complex in HPV-6/11 induced cellular proliferation. In HPV-6 and HPV-11 infected cells, expression of c-jun may be induced by inhibition of down regulation of EGF or PDGF receptors by E5 gene products [Petti et al., 1991; Conrad et al., 1993]. The constitutive growth signal is then transmitted to the nucleus where the c-jun gene is activated and up-regulated by activation of the AP-1 binding site on its own promoter [Chen, 1994b].

The infection of low-risk HPVs is also associated with a portion of low-grade CINs [Gissmann et al., 1983]. It would be interesting to know the status of c-jun expression in LR-HPV infected CINs and the role of this infection in the development and progression of this precancerous state.

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